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Renal vascular induction of TGF-β2 and renin by potassium depletion

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Renal vascular induction of TGF-β2 and renin by potassium depletion. Recently, we have found that transforming growth factor (TGF)-β2 and renin are abundantly expressed in the juxtaglomerular apparatus (JGA) of dehydrated mice. Since potassium (K^+) depletion also stimulates renin and induces hypertrophy of the JGA, we examined the ability of this maneuver to stimulate TGF-β isoforms and renin in renovascular tissue and the JGA of young rats. Sprague-Dawley rats (50 ± 5 g) were fed either a control diet or a potassium-deficient diet (<0.05% K^+) for 7, 16, or 21 days. As a control for TGF-β and renin stimulation, an additional group of animals was fed a normal diet but was water deprived for three days. Potassium-depleted animals experienced severe growth retardation but kidney weight increased significantly. Potassium depletion induced both TGF-β2 and renin immunoreactivity in renal arterioles and the JGA but had no effect on TGF-β1 and TGF-β3 isoforms. To determine the role of circulating angiotensin II in the stimulation of TGF-β2 by potassium depletion, a group of potassium-depleted rats received enalapril (100 mg/liter) in the drinking water. The addition of converting enzyme inhibitor increased both the intensity of TGF-β2 and renin staining as well as the number of cells positively stained. Our results demonstrate that K^+ depletion induces TGF-β2 and renin in renal arterioles and in the JGA. Furthermore, circulating angiotensin II is not responsible for the increase in the local expression of TGF-β2. These findings suggest that TGF-β2 may be an important mediator of JGA hypertrophy. The simultaneous induction of TGF-β2 with renin suggests that these factors may be coregulated.

An increase in the number of renin-containing cells in the juxtaglomerular apparatus (JGA) and renal arterioles has been found during development [1] and when renin synthesis is stimulated by potassium depletion, salt depletion, adrenalectomy, or by treatment with angiotensin converting enzyme inhibitors [2–6]. In adult and pediatric patients, Bartter's syndrome and renal artery stenosis [5] are associated with an increase in renin-containing cells. Under these conditions, granular cells have been identified in extraglomerular regions, such as the cortical, radial (interlobular), and arcuate arteries [1–6].

Renin secreting cells undergo hypertrophy when renin is stimulated [2, 3]. Thus, the JGA granular cells as well as renin-secreting vascular smooth muscle cells increase in size. Renin stimulation is often associated with an increase in the number of secretory granules and thickness of the afferent arteriolar wall. The molecular mechanisms responsible for these changes, however, remain unknown but suggest the presence of a co-regulated growth factor.

The vasoactive peptide angiotensin II (Ang II) is a co-mitogen for several renal mesangial cells and vascular smooth muscle cells [7–11]. Therefore, increased circulating levels of Ang II could induce myointimal hyperplasia and renal vascular smooth muscle cell growth. Angiotensin is unlikely, however, to stimulate granulogenesis and JGA hypertrophy since Ang II inhibits renin synthesis [12]. Alternatively, other growth factors, particularly TGF-β, which is induced in the JGA in the setting of severe dehydration [13], could be responsible for both hypergranulation and hypertrophy of the JGA and renal arterioles.

TGF-βs are known to modulate a number of cellular functions including proliferation, differentiation, protein synthesis, and hormonal release by several cell types [14–17]. TGF-β1 and TGF-β2 mRNAs are found in glomerular mesangial and vascular smooth muscle cells [18–19]. In addition, Ang II stimulates TGF-β1 mRNA expression in rat vascular smooth muscle cells, an effect which is thought to be responsible for the hypertrophic response of these cells to Ang II [19]. Water deprivation, which increases circulating levels of Ang II, induces accumulation of both renin and TGF-β2 in JGA of mice [13]. Water deprivation, however, is associated with changes in osmolarity, increased serum sodium and potassium concentrations, as well as increased circulating Ang II. Thus, the factors responsible for renin and TGF-β induction remain unclear.

In the present study, we investigated the effects of potassium depletion on the expression of renin and transforming growth factor β isoforms in JGA and renal arterioles of young rats. Young rats were selected because the renin-angiotensin system of young animals is particularly sensitive to changes in electrolyte imbalance. Potassium depletion stimulates circulating Ang II and induces JGA hypertrophy but, in contrast to water deprivation, does not alter serum osmolarity or sodium concentration. We compared the effects of potassium depletion with water deprivation in young rats and then explored whether circulating Ang II stimulates TGF-β by treating potassium-depleted rats with angiotensin converting enzyme inhibitors.

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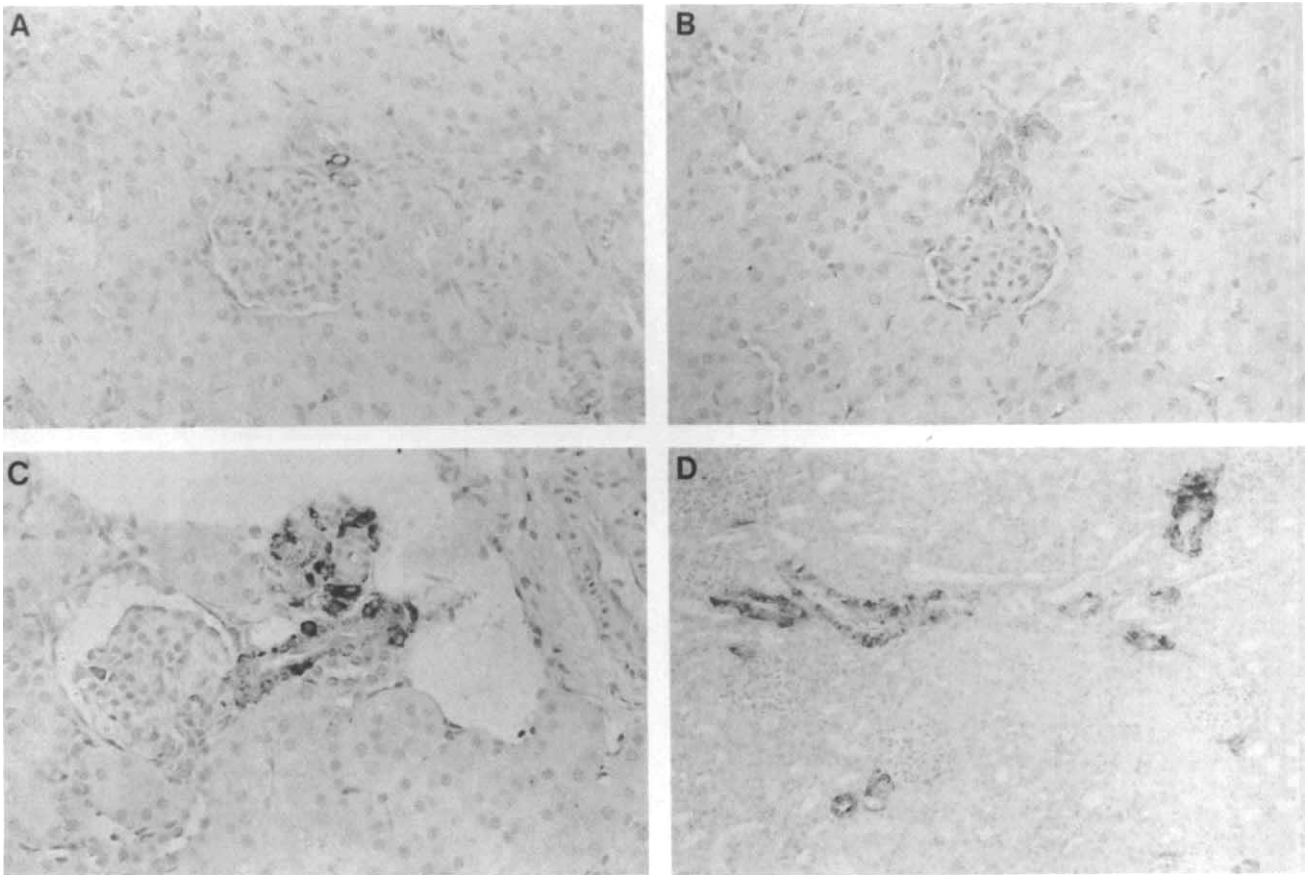


Fig. 1. *Localization of renin in rat kidney by immunohistochemistry.* **A.** Control rat kidney demonstrates staining for renin in JGA (magnification 320×). **B.** Kidney from a 16 day potassium-depleted rat demonstrates intense staining of JGA and recruitment of renin producing cells in vasculature, indicated by the arrow head (magnification 320×). Kidney from a potassium-depleted rat treated with enalapril demonstrates a marked increase in renin staining, an increase in the size of renin secreting cells, and recruitment of immunoreactive cells in renal arterioles (**C.** magnification 320×; **D.** magnification 160×).

We found that potassium depletion stimulated renin and TGF-β2 in the JGA. Induction of TGF-β2 was not dependent upon circulating levels of Ang II. In fact, inhibition of Ang II increased the expression of renin and TGF-β2 at these sites. When angiotensin converting enzyme was inhibited in the presence of potassium depletion, juxtaglomerular hypertrophy was more apparent. These findings suggest that TGF-β2 may be an important mediator of JGA hypertrophy, and that TGF-β2 and renin may be coregulated.

Methods

Experimental design

Male Sprague-Dawley rats, 50 ± 5 grams body weight, were purchased from Taconic Farms (Germantown, New York, USA). Animals were housed individually in a temperature controlled room (24°C) with a 12 hour on/12 hour off lighting schedule. Three groups of 5 animals each were studied. All rats were given food and water *ad libitum*. Group 1 was fed a standard diet containing 0.3 g% NaCl and 0.5 g% potassium. Group 2 was fed a potassium-deficient diet containing <0.05 g% K^+ and 0.3 g% NaCl. This diet is known to stimulate renin synthesis [20–22]. Group 3 was fed the same potassium-deficient diet but enalapril maleate (Merck Sharp & Dohme, Rahway, New Jersey, USA) 100 mg/liter was added to the drinking water. Standard and modified rat chow were obtained from ICN Nutritional Biochemical (Cleveland, Ohio, USA). Kidneys were harvested at 7, 16, and 21 days for immunohistochemical analysis. Since water deprivation is known to induce TGF-β2 in JGA and renal vasculature of adult mice [13], we water deprived an additional group of young rats of similar age and weight for three days as a positive control group.

Plasma samples were collected in ice-cold heparinized tubes to measure plasma electrolytes and osmolarity in treated rats. EDTA-containing tubes were used to collect samples for the measurement of plasma renin activity. Sodium and potassium concentration were measured using a KNA 2 Sodium-Potassium analyzer (Radiometer, Copenhagen, Denmark). Plasma renin activity was determined by radioimmunoassay [23]. Serum osmolarity was measured using an Advanced Digit Matic osmometer model 3 DII (Advanced Instruments, Needham Heights, Massachusetts, USA).

Antibodies

Affinity-purified rabbit polyclonal antibodies directed against peptide sequences unique to TGF-β1, TGF-β2 and TGF-β3 were prepared, and have been shown previously to have

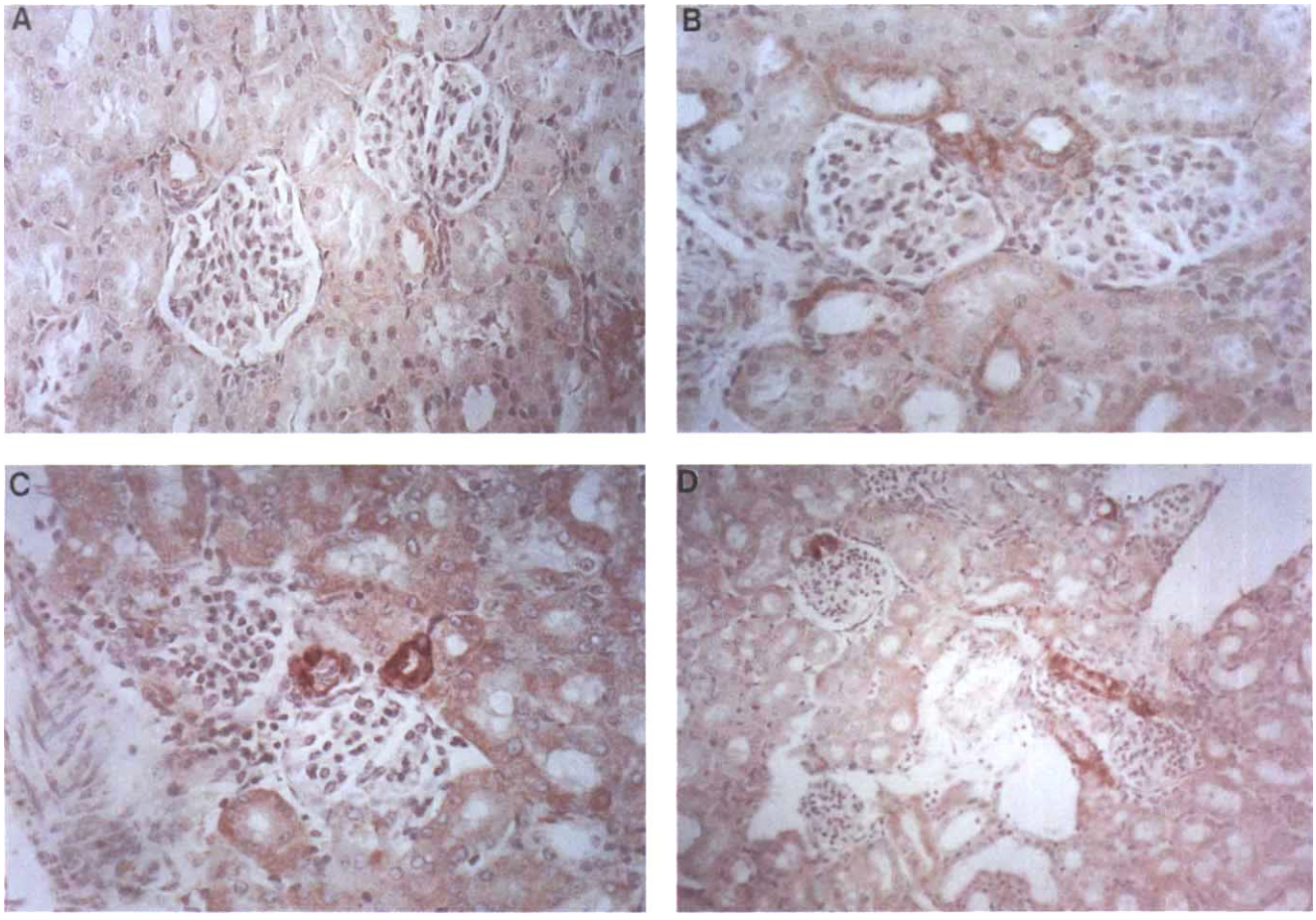


Fig. 2. *Localization of TGFβ_2 in rat kidney by immunohistochemistry.* **A.** Control rat kidney demonstrates faint staining in occasional cortical tubular cells and no staining in JGA or renal arterioles (magnification 320×). **B.** Kidney from a 16 day potassium-depleted rat demonstrates faint staining in cortical tubular cells and localized staining in JGA and renal arterioles (magnification 320×). **C.** Kidney from a potassium-depleted rat treated with enalapril demonstrates intense staining in JGA (magnification 320×) and recruitment of TGFβ_2 immunoreactive cells along renal arterioles (**D**: magnification 160×).

isoform specificity [24–26]. The peptide sequences were from the latency-associated peptide of the TGF-β1 precursor and from the mature forms of TGF-β2 and TGF-β3. Anti-rat renin polyclonal antibody was a gift of Dr. T. Inagami (Vanderbilt University, Nashville, Tennessee, USA). Antiserum was diluted 1:2500 in PBS containing 3% BSA. The specificity and characteristics of this antibody have been reported previously [27, 28].

Histochemistry

Kidneys were weighed, fixed in zinc formalin (Anatec, Battle Creek, Michigan, USA), and embedded in paraffin. TGF-β isotypes were localized in 5 μm sections using an avidin-biotin-peroxidase kit (Vector Laboratories, Inc., Burlingame, California, USA). Deparafinized sections were digested with hyaluronidase (1 mg/ml, Calbiochem-Boehring Corp., La Jolla, California, USA) and blocked with 5% normal goat serum, 1% BSA fraction V (Miles Laboratories Inc., Naperville, Illinois, USA) and 1% ovalbumin (Fluka, Inc., Ronkonkoma, New York, USA). Sections were then incubated with primary antibodies (4 to 10 μg/ml for TGF-β antibodies or 1/5,000 dilution of anti-renin antiserum) in Tris-buffered saline (TBS) containing 1% BSA for 16 hours at 4°C. Sections were washed in TBS/0.1% BSA and then incubated with biotinylated goat anti-rabbit IgG followed by avidin-biotin complex. The sections were incubated in 0.5 mg/ml 3,3′-diaminobenzidine (Sigma Chemical Co., St Louis, Missouri, USA) and in 0.1% hydrogen peroxide for three to five minutes and counterstained with Mayer's hematoxylin. As a control, the first antibody was omitted and replaced by an equivalent concentration of nonimmune rabbit IgG. The specificity of the primary TGF-β2 antibody has been demonstrated previously by complete blocking in the presence of a 20-fold molar excess of the immunizing peptide [13].

Statistical analysis

Results were expressed as mean ± SEM and significance was analyzed by analysis of variance. P values of less than 0.05 were considered statistically significant.

Results

After two weeks, the two groups of rats fed a potassium-depleted diet weighed less than control animals: control animals weighed 108 ± 7 g, potassium-depleted rats weighed 65 ± 8 g, and potassium-depleted rats treated with enalapril weighed 62

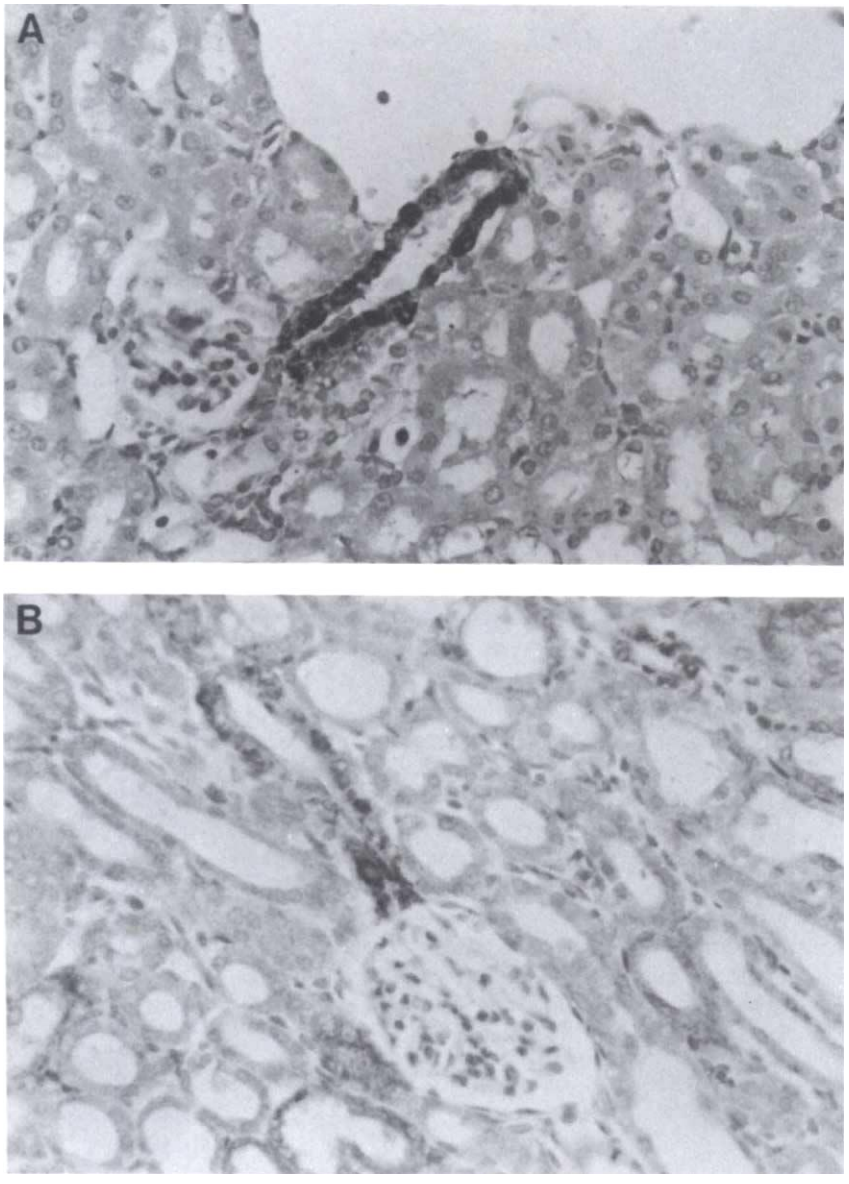


Fig. 3. *Colocalization of renin and TGF-β2 in vascular tissue.* In potassium-depleted animals treated with enalapril, recruitment of cells that were identified by both renin (**A**) and TGF-β2 (**B**) immunoreactivity could be found along renal arterioles (magnification 640×).

± 6 g, respectively ($P < 0.05$, potassium-depleted vs. control). In contrast to body weight, there was no significant difference in absolute kidney weight between the three groups: control 0.51 ± 0.05 g, potassium-depleted 0.47 ± 0.04 g, and potassium-depleted treated with enalapril 0.48 ± 0.05 g, respectively. When the weight of both kidneys was expressed as a percentage of body weight, however, kidney weight was significantly increased in the potassium-depleted groups (control 0.47%, potassium-depleted 0.72%, and potassium-depletion treated with enalapril 0.77%).

As expected, serum potassium was decreased in potassium-depleted animals when compared to control animals: 2.3 mEq/liter ± 0.1 versus 4.4 ± 0.1 mEq/liter. Enalapril treatment of potassium-depleted rats produced an intermediate serum potassium concentration of 3.5 ± 0.4 mEq/liter, presumably from the loss of angiotensin-stimulated aldosterone secretion. Serum sodium and osmolarity were not significantly different between all groups: (serum sodium in control 139 ± 0.9, in potassium depletion 136 ± 1.2, and in potassium depletion plus enalapril 137 ± 1.3 mEq/liter; serum osmolarity in control 298 ± 1.5, in potassium depletion 293 ± 2.1, and in potassium depletion plus enalapril 294 ± 3.1 mOsm/liter). In contrast, water deprivation significantly increased serum potassium (6.4 ± 0.3 mEq/liter), serum sodium (152 ± 2 mEq/liter), and serum osmolarity (309 ± 2 mEq/liter) when compared to all other groups.

As has been shown previously, potassium depletion induced an increase in plasma renin activity. Potassium depletion alone increased plasma renin activity sixfold, and when combined with enalapril treatment, plasma renin activity increased eightfold above control. Plasma renin activity of rats drinking water *ad libitum* was 2.7 ± 0.4 ng/ml/hr, plasma renin activity of potassium-depleted rats was 17.3 ± 2 ng/ml/hr, and plasma renin activity of potassium-depleted rats receiving enalapril in the drinking water was 25 ± 4 ng/ml/hr ($P < 0.05$). Water

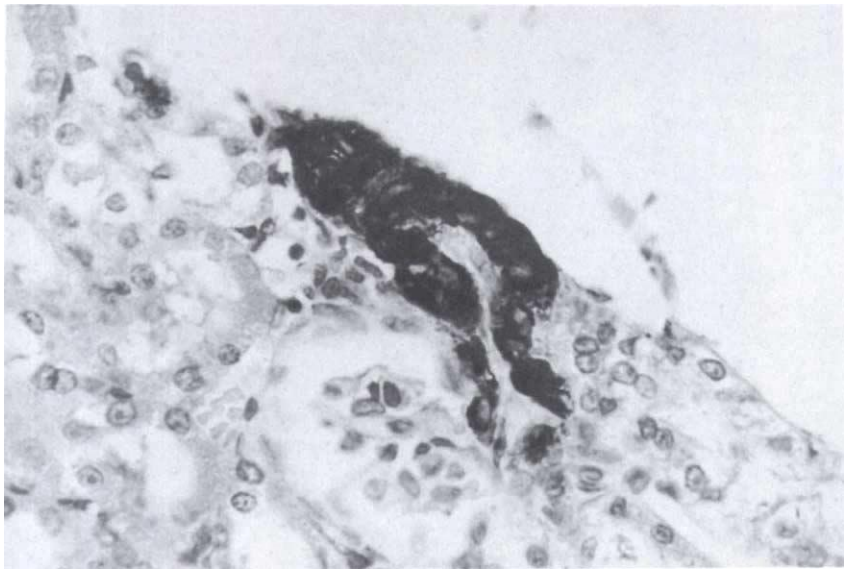


Fig. 4. *Localization of renin in rat kidney by immunohistochemistry (magnification 640×).* Hypertrophy of the JGA from a potassium-depleted enalapril-treated rat. JGA cells are increased in size and renin content.

deprivation increased plasma renin activity threefold (9.2 ± 1 ng/ml/hr) when compared to control rats (2.7 ± 0.4 ng/ml/hr; $P < 0.05$).

To localize the sites of immunoreactive renin within kidney, renal tissue was examined by immunohistochemistry using anti-renin antibodies (Fig. 1). In control animals, immunoreactive renin staining was limited to the juxtaglomerular cells (Fig. 1A). In potassium-depleted rats, increased renin staining was detected in the JGA (Fig. 1B), but, in addition, potassium depletion increased the number of renin immunoreactive cells in renal arterioles as well (arrow). Dehydrated rats showed JGA renin staining similar to that of potassium-depleted rats (data not shown). Treatment with enalapril dramatically increased the number of renin immunoreactive cells in JGA and along renal arterioles (Fig. 1 C and D).

At all time points, distribution of TGF-β2 was similar to that of renin following potassium depletion (Fig. 2). The intensity of renin and TGF-β2 immunostaining correlated directly with the duration of treatment. Increased renin and TGF-β2 were detected after seven days and maximal immunostaining was present after 21 days (data not shown). No significant staining was present in JGA or vascular smooth muscle cells of control animals (Fig. 2A). Tubule epithelial cells from control animals, however, exhibited faint staining with anti-TGF-β2 antibody. Potassium depletion induced a significant recruitment of cells that were immunoreactive for TGF-β2 in the JGA (Fig. 2B). Dehydrated rats showed similar staining in JGA (data not shown). Potassium-depleted animals treated with enalapril had further recruitment of immunoreactive cells in the JGA and smooth muscle cell layer of periglomerular renal arterioles (Fig. 2 C and D). At higher magnification, recruitment of renin (Fig. 3A) and TGF-β2 (Fig. 3B) immunoreactive cells along renal arterioles was clearly visible in potassium-depleted rats treated with enalapril. Furthermore, potassium depletion in combination with enalapril induced marked juxtaglomerular hypertrophy (Fig. 4).

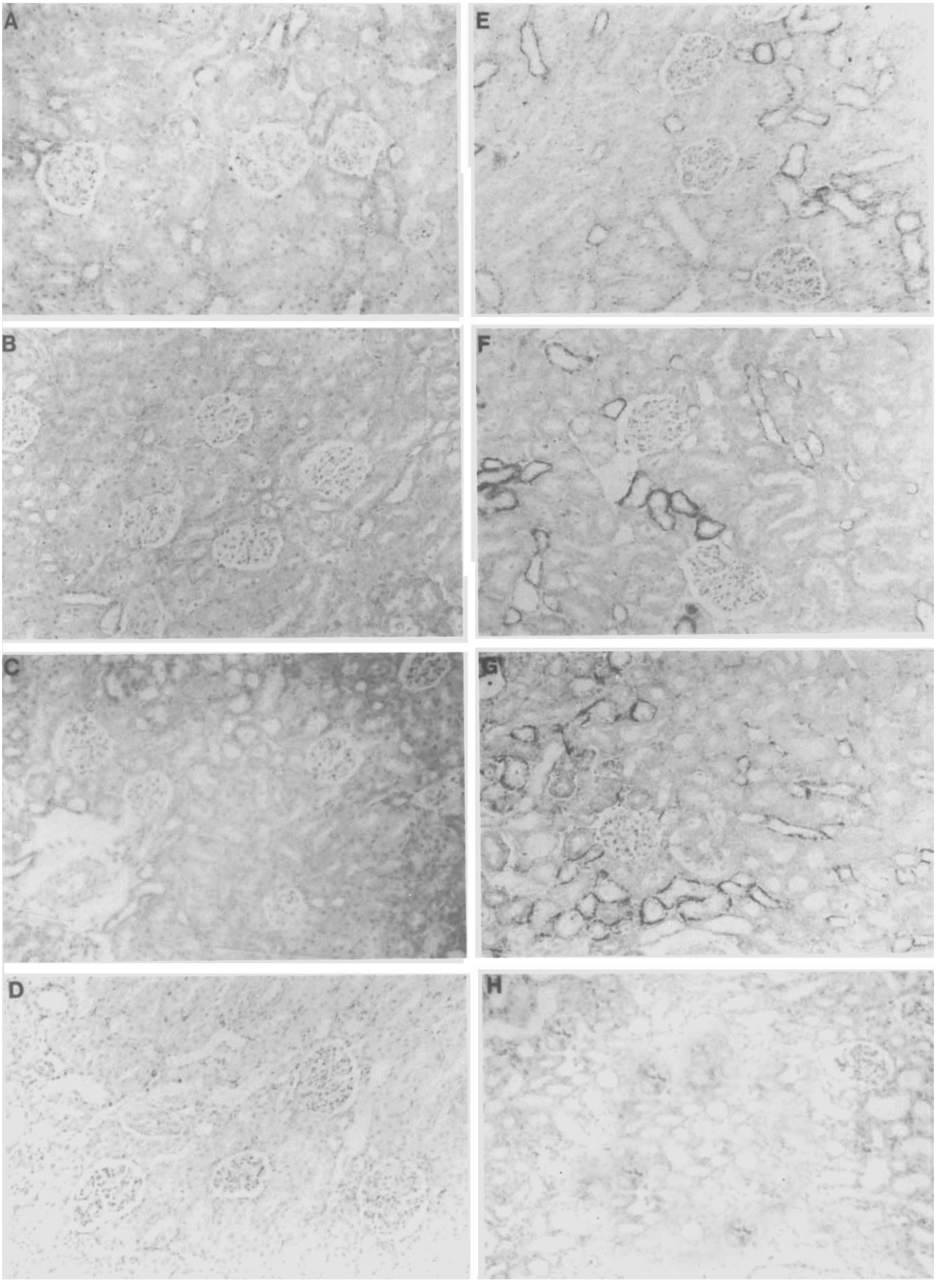
In contrast to the clear differences in localization of TGF-β2, the staining patterns with anti-TGF-β1 and anti-TGF-β3 antibodies were heterogeneous, with some tubules demonstrating focal areas of slightly greater reactivity than others. These changes were highly variable and no clear pattern emerged. In all groups, staining of tubular epithelial cells with TGF-β1 antibody was generally diffuse with some focal areas of greater staining (Fig. 5 A, B, and C). A similar staining pattern was detected with anti-TGF-β3 antibody (Fig. 5 E, F, and G).

Discussion

Potassium depletion induces a complex physiologic response to retain potassium and maintain intracellular electrolyte integrity. Increasingly, potassium deficiency has been recognized to produce other responses that may contribute to disease including an increase in systemic blood pressure [29], stimulation of plasma renin activity [2], and juxtaglomerular hypertrophy [3]. The molecular mechanisms responsible for these changes, however, remain obscure.

In the present study, we demonstrated that potassium depletion or water deprivation induced accumulation of TGF-β2 and renin in juxtaglomerular cells and renal arterioles in young rats. In contrast to the clear colocalization of TGF-β2 with renin in JGA and renal arterioles, immunostaining for TGF-β1 and TGF-β3 in renal tubules was not significantly different between control and treated animals. Water deprivation was associated

Fig. 5. *Localization of TGF-β1 and TGF-β3 expression in rat kidney by immunocytochemistry.* Immunoreactive TGF-β1 can be detected in a heterogeneous pattern in tubular epithelial cells of (**A**) controls, (**B**) potassium-depleted animals, and (**C**) potassium-depleted animals treated with enalapril (magnification 160×). In all groups, some cortical tubular cells stained more intensely than others. Control nonimmune serum IgG was negative (**D**). Immunoreactive TGF-β3 was found in the renal tubular epithelial cells in (**E**) control, (**F**) potassium-depleted, and (**G**) potassium-depleted animals treated with enalapril (magnification 160×). Staining was absent in potassium-depleted animals treated with nonimmune serum IgG (**H**). Some cortical cells were stained more intensely than others.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with an increased in serum osmolarity, sodium and potassium concentration, and circulating Ang II level. In contrast, potassium depletion induced a different physiologic response without significantly changing serum osmolarity and sodium concentration. Furthermore, no significant kidney growth was detected after three days of water deprivation, whereas potassium depletion induced both kidney growth and JGA hypertrophy. Despite the different physiologic responses associated with renin stimulation, the pattern of simultaneous induction of renin and TGF-β2 expression in JGA and vascular smooth muscle remained.

Potassium depletion and dehydration both increase plasma renin activity and circulating levels of Ang II. Since Ang II induces TGF-β expression as well as vascular smooth muscle cell hypertrophy, it is possible that the observed responses to potassium depletion could be mediated by Ang II alone. To exclude this possibility, we treated potassium-depleted animals with an angiotensin converting enzyme inhibitor to decrease circulating levels of Ang II. We failed to detect a reduction in immunostaining for TGF-β2, and angiotensin converting enzyme inhibition was actually associated with greater recruitment of TGF-β2 and renin immunoreactive cells as well as marked juxtaglomerular hypertrophy. It is likely that converting enzyme inhibition induces several other confounding factors that may also affect cell growth and renin and TGF-β2 synthesis including kinins and products of arachidonic acid metabolism. In summary, however, this finding suggests that circulating Ang II does not mediate the effects of potassium depletion on TGF-β2 production, and strongly suggests that TGF-β2 and renin are coregulated in rodents.

Ang II is known to stimulate smooth muscle cell growth during development and after vascular injury [8, 30]. It has been postulated that the effects of Ang II on smooth muscle cell growth are mediated by TGF-β [31]. We have demonstrated, however, that angiotensin converting enzyme inhibitors further stimulate TGF-β2 through a mechanism that is distinct from Ang II. Since accumulation of TGF-β2 may potentiate the hypertrophy response of renal vascular tissue, it is possible that the use of angiotensin converting enzyme inhibitors under these circumstances may not be beneficial and may actually be harmful.

Previous studies have demonstrated that TGF-β2 mRNA is present in vascular smooth muscle cells, granular cells from the JGA, and renal mesangial cells [18–19, 32]. Thus, increased production of TGF-β2 at these sites most likely represents increased local synthesis. Immunoreactive TGF-β2 observed in JGA and smooth muscle cells, however, may represent the accumulation of this growth factor from reduced secretion or increased uptake of TGF-β2 from distal sites. Further studies to localize TGF-β2 mRNA by *in situ* hybridization will help to clarify this issue.

The remarkable colocalization of TGF-β2 and renin during dietary potassium depletion strongly suggests a regulatory interaction. Although speculative, the presence of TGF-β2 in renin-producing cells suggests that TGF-β2 may induce hypertrophy of renin producing cells in smooth muscle and the JGA, and may contribute to the differentiation of cells along the renal arteriole into a renin secreting phenotype. Indirect support for this hypothesis comes from several laboratories. TGF-βs appear to play an important role in the development of vascular smooth muscle hypertrophy in other conditions such as hypertension [19, 33, 34]. Since renin is synthesized, stored, and released from differentiated smooth muscle cells, TGF-β may also increase granulogenesis of renin secreting cells. TGF-β peptides, for example, stimulate renin release in the adrenal gland [35] and TGF-β can regulate other hormonal systems as well [15–17]. Thus, TGF-β is an excellent candidate for the growth factor responsible for hypertrophy of renin-secreting cells as well as the growth factor responsible for differentiation of non-renin secreting vascular smooth muscle into a renin-secreting cell.

In conclusion, we have demonstrated that potassium deficiency induces the expression of renin and TGF-β2 in JGA and along renal arterioles. This effect of potassium deficiency is enhanced by angiotensin converting enzyme inhibition. These findings suggest that TGF-β2 may be an important modulator of JGA function and renal vascular cell growth and may well affect renin synthesis and secretion as well. The colocalization of renin with TGF-β2 suggests that both systems interact in potassium homeostasis and that TGF-β2 may have an as yet undefined role in maintaining electrolyte balance. Furthermore, vascular TGF-β2 expression, like renin, may be developmentally regulated and participate as a development growth factor for renin-secreting cells [36, 37]. Thus, renin and TGF-β2 may have complementary roles during kidney growth, vascular development, and disease.

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